R55-6

Reprinted from SURGERY, St. Louis.
Vol. 37, No. 6, Pages 935-946, June, 1955. (Printed in the U. S. A.)

THE EFFECT OF PREVIOUS LEVEL OF PROTEIN FEEDING ON WOUND HEALING AND ON METABOLIC RESPONSE TO INJURY

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INTRODUCTION

THE importance of protein nutrition in wound healing is widely recognized. Frank protein deficiency leads to delay in wound healing, but satisfactory healing is ultimately attained (see Levenson and associates[1] for review of literature). In the uninjured animal the size of the body stores of protein is influenced by the amount of protein in the diet. As the protein level of the diet is increased above that required for nitrogen equilibrium additional amounts of protein are stored, especially in the liver.[2] Because this extra protein storage induced by high protein feeding is readily reversed by caloric restriction, starvation,[2, 3] or injury,[4] it has come to be designated as "labile" protein stores. Conversely the magnitude of the catabolic response to injury, in regard to nitrogen loss, is proportional to the size of the "labile" protein stores and therefore to the preinjury plane of protein feeding. The question presents itself as to whether this metabolic variation in response to injury induced by differences in preinjury protein feeding is associated with differences in wound healing. It was therefore undertaken in this study to compare the response to injury of animals previously fed minimal or liberal levels of dietary protein.

METHODS

Male albino rats were fed a commercial stock diet until 250 to 300 grams of body weight was attained. The animals were then divided into three groups and, for two weeks, fed purified diets which varied only in the amount of protein supplied: one, two, or three times the minimum requirement for nitrogen equilibrium (53 mg. nitrogen per 100 grams of body weight per day[5]). The protein source was casein supplemented with methionine, 30 mg. per gram of protein, incorporated at levels 10.45 per cent to 31.34 per cent of the diet. Other diet components were: vitamin-supplemented corn oil,* 10 per cent; U.S.P. salts No.

The opinions expressed in this paper are those of the authors and do not necessarily represent those of any governmental agency.

Received for publication Dec. 23, 1954.

*Containing 0.01 per cent halibut liver oil and 0.003 per cent α tocopherol.

2, 4 per cent; B vitamin mixture,* 1 per cent; and sucrose 53.66 per cent to 74.55 per cent. Forty-eight calories was offered per day, which was sufficient to maintain body weight essentially constant, and the feeding period was limited to seven hours. Urine and feces were collected during the last two days of this period for measurement of nitrogen balance.

On the fifteenth day five control animals from each group were sacrificed and the remainder subjected to injury. The animals to be injured were first anesthetized with sodium pentobarbital and shaved. A 4 cm. midline abdominal incision was then performed and the wound closed by interrupted, through-and-through, No. 34 steel sutures at 1 cm. intervals. A 5 cm. shaved dorsal area, immediately anterior to the transverse process of the sacrum, was marked and the animal burned within the demarcated space by immersion for fifteen seconds in water heated to 90° C., a slight modification from the procedure of McCarthy.[6] The incision served as a means of quantitating wound healing; the burn was added as a further metabolic stress. Levenson and co-workers[7] found that incised wounds healed more slowly in burned animals than in unburned controls.

No food was given on the day of injury, but ad libitum water intake was permitted. During the recovery period the groups prefed one or three times the protein requirement were divided, one half of the animals continuing on the same diet and one half receiving two times the protein requirement. This arrangement, as shown in Table I, provided three groups which differed only in the diet fed before injury and three groups in which the diet pattern was unchanged but at varying levels. On the first day after injury two-thirds of the previous food intake was offered, and during the remainder of the recovery period the standardization allowance was supplied, 48 calories per day. Urine collections were made over two-day periods beginning with the day of injury and continuing to the tenth day postinjury. Five or six animals from each group were sacrificed on the fourth, seventh, and tenth postinjury days.

Table I. Nitrogen Content of Food

GROUP	NITROGEN ALLOWANCE			
	STANDARDIZATION		POSTINJURY	
	MG./DAY	% OF REQUIREMENT	MG./DAY	% OF REQUIREMENT
1-1	162	100	162	100
1-2	162	100	315	200
2-2	315	200	315	200
3-3	475	300	475	300
3-2	475	300	315	200

At sacrifice, the abdominal wound, burned area, liver, and adrenals were excised. One half of the wound was preserved for histologic examination and the remainder used for measurement of tensile strength as follows: A 1 cm. strip was cut at suture lines and suspended from a screw clamp parallel to the incision. A second clamp to which was attached a paper container was fastened at the opposite end while supported from below, so that no weight was borne by the tissue. The support was then removed and lead shot added at a constant rate until the wound separated. The tensile strength was the combined weight of clamp plus container plus shot. The procedure was carried out on two strips from each wound (one from each end) and the average value used for comparisons.

*Supplying per 100 Gm. of diet:

thiamine hydrochloride	0.50 mg.	p-aminobenzoic acid	5.00 mg.
riboflavin	0.50 mg.	biotin	0.01 mg.
pyridoxine hydrochloride	0.25 mg.	folic acid	0.10 mg.
nicotinamide	2.00 mg.	vitamin K	0.10 mg.
calcium pantothenate	2.00 mg.	choline chloride	100.00 mg.
inositol	10.00 mg.	vitamin B_{12}	50 μg

The burns were inspected for estimation of necrosis, ulceration, and suppuration and the area traced on acetate film. The tracing was cut out and weighed and the area of the burn calculated from the weight of a known area of the acetate film. The body surface area of the animal was calculated from the formula derived by Mitchell[8]: body surface area, sq. cm. = 13.2 weight.$^{0.64}$ Burn size was then expressed as a percentage of body surface. A representative portion of the burn was reserved for histologic examination.

The adrenals were cleaned and weighed immediately. The livers were weighed and analyzed for moisture, nitrogen, and fat.[9]

The methods used for staining and examining the slides of the wounds were those described in detail by Pirani, Stepto, and Sutherland.[10]

The wound sections were examined in a uniform manner without previous knowledge of the origin of the slide. The wound area was arbitrarily defined as that limited by the epidermis and the peritoneal surface in one direction and by the width of two low-power (X35) fields on the other. Each specific histologic detail being investigated was then graded in arbitrary units (1+ to 4+) and tabulated in an attempt to obtain a quantitative evaluation of the possible changes. The general appearance, as well as the cellular features of the wounds, were studied in the slides stained with hematoxylin and eosin. Collagen fibers were stained by the van Gieson technique.[11] As the collagen fibers are formed and mature, they gradually vary in color from pale pink to brilliant red, while immature collagen stains uniformly yellow. Toluidine blue for mucopolysaccharides was used in a 1:1,000 aqueous solution at pH 4.25 for one minute.[12] After staining, the section was washed in distilled water for twenty seconds, blotted dry with filter paper, and mounted in crown oil. The slides were read within five minutes. The leukofuchsin periodic acid method for glycoprotein[13] disclosed leukofuchsin-reacting material in small amounts distributed throughout the wound granulation tissue and within mast cells.

RESULTS AND DISCUSSION

Body Weight and Food Consumption.—At the end of standardization the body weight of the group fed the minimal protein level (270 grams) was significantly lower than that of the other two groups (279 grams) ($F = 13.13$, $P<0.01$). On the day of wounding the differences between the subgroups within each level of preinjury protein feeding were not significant ($F<1$).

The mean body weight loss between the day of wounding and the day of sacrifice was 10.2 grams at PI 4 (postinjury day 4), 11.7 grams at PI 7, and 12.5

grams at PI 10. These differences are not significant ($F = 1.29$, $P>0.05$). Nor were there significant differences in weight loss among the five postinjury diet groups ($F = 1.89$, $P>0.05$). The body weight fell sharply in the first two postinjury days, rose somewhat in the next two days, and then remained relatively constant.

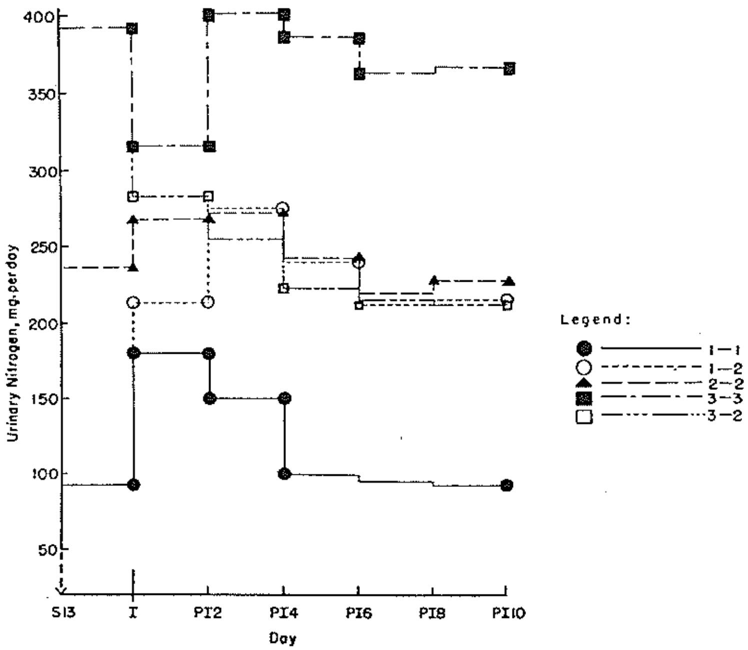

Fig. 1.—Urinary nitrogen excretion pre- and postinjury.

The total caloric intake between the day of injury and the day of sacrifice did not differ significantly between the various diet groups (PI 4, $F < 1$; PI 7, $F = 1.42$; PI 10, $F < 1$). In the PI 10 subgroups used for nitrogen excretion measurement throughout, slight variation in food consumption was noted and is discussed in connection with nitrogen balance.

Urinary Nitrogen Excretion and Nitrogen Balance (Figs. 1, 2, and 3).—During the last two days of the period of standardization all groups were in positive nitrogen balance and there were no significant differences between groups in this regard. When the minimum protein requirement was met, 56 mg. of nitrogen was retained per day; at two or three times requirement, daily retentions were 62 mg. to 64 mg. Virtually all the additional nitrogen fed was excreted in the urine, the daily excretion rising from 92 mg. to 236

mg. to 392 mg. with increasing intake (Fig. 1). The wasteful nature of the excess feeding is best expressed by converting the nitrogen retentions to percentage of that absorbed, giving values of 38 per cent, 21 per cent, and 14 per cent at one, two, and three times the requirement, respectively.

The pattern of urinary nitrogen excretion is shown in Fig. 1. On the day of injury and the first day following, nitrogen excretion of the groups prefed the minimum requirement (1-1 and 1-2) rose markedly above the preinjury level. Those animals prefed double the requirement (2-2) exhibited only a slight rise in nitrogen excretion, and those previously maintained at three times requirement (3-3 and 3-2) excreted less than during the control period.

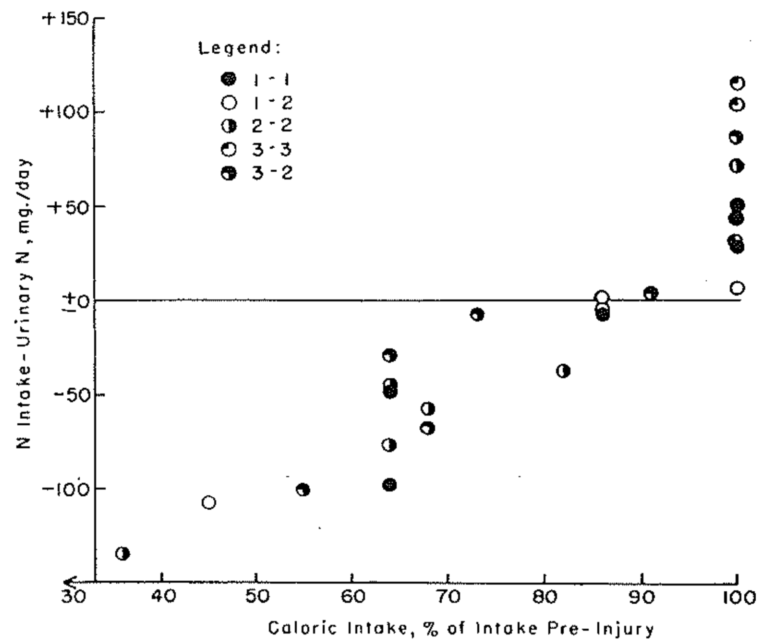

Fig. 2.—Relationship between food intake and nitrogen balance at the second and third days after injury.

During the next period the results were confounded by incomplete food consumption but three distinct levels of excretion related to intake were apparent. By the fourth and fifth days after injury excretions approximated those obtained before injury and remained essentially constant thereafter. The group continued at the minimum protein level returned to excretions identical with those found during standardization, but excretions at two or three times minimal requirement were slightly less than previously observed. The catabolic response to injury cannot be accurately assessed because the food intake in the immediate postinjury period was less than during the standardization

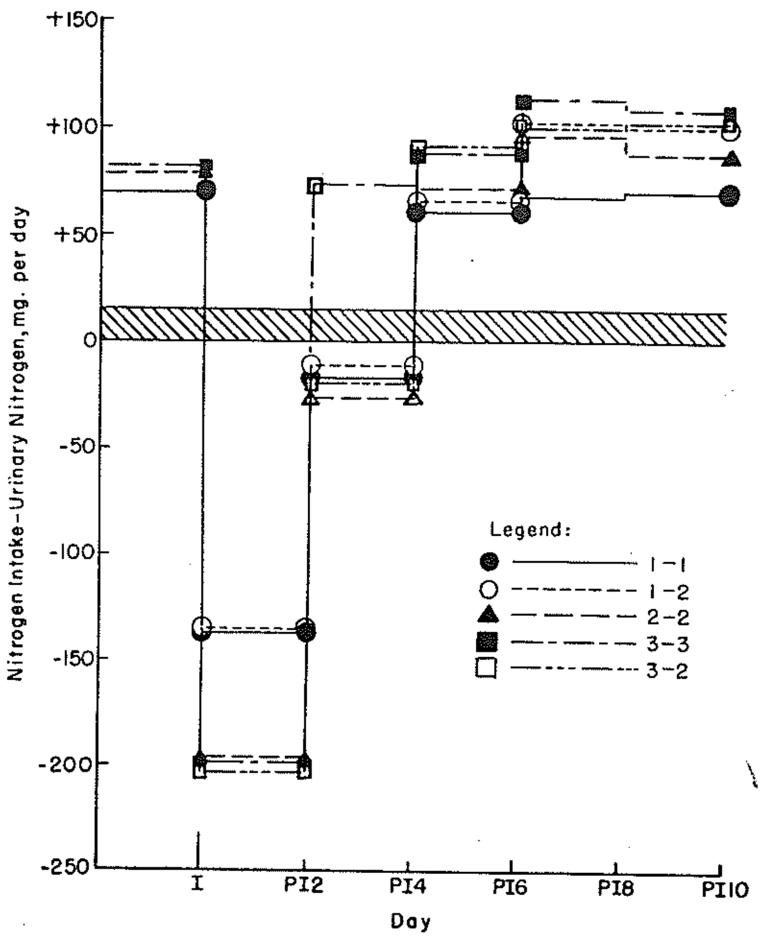

Fig. 3.—Nitrogen "balance" pre- and postinjury.

period, so that the effects of decreased food intake and response to injury were confounded. The total catabolic response to these two effects, as reflected by negativity of nitrogen balance, will be considered presently. However, by the

time food intake had returned to the standardization level (PI 4 and 5) nitrogen excretion was not greater than during standardization. Therefore, the catabolic response to injury, if present, had ended by this time.

The marked effect of food intake on nitrogen balance, during the second and third days after injury, is demonstrated in Fig. 2. Regardless of protein supplied, nitrogen balance was negative unless the food intake, and hence caloric intake, was above 85 per cent of that required to maintain body weight before injury. Comparisons between groups during the second and third days after injury could not be made because of this variation in food consumption. Only the animals given the highest protein level ate the total food allowance during this period, and they were in positive balance.

Immediately following injury the groups prefed two or three times the minimum requirement were in more negative nitrogen balance than were those on minimal allowance: –200 mg. per day as opposed to –137 mg. per day ($F = 29.55$, $P < 0.01$). The difference between the group prefed twice the minimal nitrogen requirement and the groups prefed three times this level was not significant ($F < 1$). From the fourth day postinjury on, all groups were in positive nitrogen balance and those fed the luxus levels of protein retained more nitrogen (95 mg. per day) than the low-protein group (65 mg. per day) ($F = 18.78$, $P < 0.01$), but the difference between the groups on two and three times minimal requirement was not significant ($F = 3.76$, $P > 0.05$). On the higher-protein diets greater retention of nitrogen was attained during recovery than preinjury ($F = 5.56$, $P < 0.05$), but retention of the lower-protein diet was unchanged. The nitrogen balance of the 1-2 group was significantly greater than that of the 1-1 group ($F = 8.07$, $P < 0.01$), but the retention of the 3-2 group was not significantly greater than that of the 1-2 group ($F = 1.25$, $P > 0.05$), nor was that of the 3-3 group superior to that of the 3-2 group ($F < 1$). It would appear, from the standpoint of nitrogen balance, that the most efficient scheme of feeding was the minimum requirement before injury with an increased intake to double the requirement during recovery. The positivity of balance rose significantly in all diet groups between PI 4 and 5 and PI 6 and 7 ($F = 23.33$, $P < 0.01$) but did not change significantly between PI 6 and 7 and PI 8 and 9 ($F < 1$).

Liver Weight and Composition (Tables II and III).—At the end of standardization the means of the wet weight of the liver did not differ significantly among the groups in the experiment as a whole ($F < 1$). In the postinjury period mean wet weight of the liver did not differ significantly among the diet groups ($F < 1$) or among the groups sacrificed on various days ($F = 2.305$, $P > 0.05$). Similarly the total water content of the liver did not differ among diet groups at the end of standardization ($F < 1$) or in the postinjury period among diet groups ($F < 1$) or among groups sacrificed on various days ($F = 2.72$, $P > 0.05$). However, the wet weight of the liver and percentage of water increased slightly in all groups after injury. Only in the groups prefed triple the protein requirement were these increases significant (P < 0.01), and the increase in weight was accountable to the increase in total water content.

TABLE II. LIVER WEIGHT AND MOISTURE CONTENT

GROUP	1-1	1-2	2-2	3-3	3-2
Wet weight					
control, grams	14.33		14.68	13.60	
PI 4, grams	14.96	14.78	14.98	16.10	15.07
PI 7, grams	13.79	14.80	14.44	14.45	14.31
PI 10, grams	13.99	14.85	14.35	15.13	15.44
Moisture					
control, %	72.4		70.1	69.8	
total, grams	10.37		10.32	9.49	
PI 4, %	74.0	73.7	73.2	73.0	73.0
total, grams	11.07	10.90	10.97	11.75	11.00
PI 7, %	72.7	73.6	72.8	73.2	72.9
total, grams	10.1	10.91	10.52	10.59	10.42
PI 10, %	73.4	73.4	72.8	72.6	73.3
total, grams	10.27	10.89	10.44	10.98	11.33

TABLE III. LIVER NITROGEN AND FAT CONTENT

GROUP	1-1	1-2	2-2	3-3	3-2
Nitrogen					
control, %*	11.41		11.70	12.04	
total, mg.	451		510	494	
PI 4, %	12.06	12.49	12.19	12.55	12.35
total, mg.	469	484	490	545	502
PI 7, %	12.37	12.71	12.72	12.74	12.73
total, mg.	467	496	499	492	494
PI 10, %	11.63	12.53	12.31	12.55	12.25
total, mg.	432	496	481	520	510
Fat					
control, %*	8.16		8.81	8.18	
total, mg.	321		384	337	
PI 4, %	8.30	7.31	8.39	7.00	7.82
total, mg.	322	283	332	305	315
PI 7, %	7.79	6.83	7.17	7.51	6.90
total, mg.	293	266	282	286	266
PI 10, %	8.49	7.29	7.54	7.16	7.87
total, mg.	313	290	294	297	328

*Dry weight basis.

The total nitrogen content of the liver at the end of standardization was higher in the groups receiving two or three times the minimal requirement than in the group receiving the minimal level (F = 4.24, P < 0.05). In the postinjury period the differences attributable to day of sacrifice were not significant (F < 1). The liver nitrogen content of the 1-1 group was significantly lower (F = 9.31, P < 0.01) and of the 3-3 group significantly higher (F = 5.26, P < 0.05) than those of the other groups, indicating that the extremes of levels of protein feeding were reflected in liver nitrogen stores during recovery.

The total fat content of the liver did not differ significantly among the diet groups at the end of standardization (F = 3.58, P > 0.05), nor did the diet groups differ significantly in the postinjury period (F = 1.69, P > 0.05).

However, the liver fat content in the postinjury period was significantly lower than at the end of standardization in all diet groups (F = 26.73, P < 0.01). In each diet group the lowest liver fat content occurred on the seventh day postinjury, and the value for this day differed significantly from the PI 4 and PI 10 values (F = 13.37, P < 0.01), but the PI 4 and PI 10 values did not differ significantly (F < 1).

Adrenal Weight (Table IV).—There were no significant differences in adrenal weights prior to injury between groups fed the different protein levels (F < 1). Adrenal hypertrophy occurred in all groups during the postinjury period (F = 37.09, P < 0.01). During the postinjury period the differences between days of sacrifice (F < 1) and between diets (F = 1.05, P > 0.05) were not significant, but the diet-days interaction was highly significant (F = 3.14, P < 0.01). This indicates that the pattern of change in adrenal weight in the course of the postinjury period varied greatly among the diet groups, as is evident from the data. Thus groups 1-1, 3-3, and 3-2 showed the greatest adrenal size by PI 4, whereas in groups 1-2 and 2-2 this occurred on PI 10, at which time the adrenals of the other groups had returned to nearly normal size. This pattern does not suggest any simple relationship between the time course of adrenal enlargement after injury and the level of protein feeding before or after injury.

TABLE IV. ADRENAL WEIGHT

GROUP	1-1	1-2	2-2	3-3	3-2
Control, mg./100 grams BW*	15.3		16.2	16.5	
PI 4, mg./100 grams BW	20.6	18.0	17.9	19.9	19.2
PI 7, mg./100 grams BW	17.5	18.2	19.5	19.8	18.9
PI 10, mg./100 grams BW	17.2	19.6	19.9	18.2	17.9

*Body weight.

Under certain experimental conditions high-protein feeding has been found to lead to adrenal hypertrophy.[14] In the present study the enlargement of the adrenals may reasonably be ascribed to the injuries acting as stressor agents.[15] The cause of the difference in time course of this adrenal hypertrophy among the diet groups is not apparent.

Burn Size (Table V).—The area of the burn, expressed as per cent of body surface area, did not differ significantly among the diet groups (F < 1). There was, however, a highly significant variation in burn size on the various postinjury days (F = 9.21, P < 0.01) which was entirely attributable to the low value on PI 7 since the values on PI 4 and PI 10 did not differ significantly (F = 1.81, P > 0.05). This decrease in burn size in the intermediate period can reasonably be attributed to cicatricial contraction.

TABLE V. BURN, PER CENT BODY SURFACE AREA

GROUP	PI 4	PI 7	PI 10
1-1	6.3	5.2	6.1
1-2	7.6	5.2	6.5
2-2	7.6	5.2	6.6
3-3	6.8	5.2	6.7
3-2	6.7	5.6	6.2

In order to determine whether the variation in burn size had a significant effect on the tensile strength of the wounds, an analysis of covariance was made, using the burn size as the covariate and the tensile strength as the variate. A test for reduction of error mean square of the tensile strength values due to regression on burn size gave an F value of less than 1 (not significant). In the 15 subgroups the correlation coefficient between burn size and tensile strength was statistically significant in only one, and an F test for differences among subgroups in regression of the variate on the covariate gave a value of less than 1 (not significant). On the basis of these analyses it was concluded that within the range of burn size encountered in this study there was no correlation between burn size and tensile strength of the wound.

Tensile Strength of Wounds (Table VI).—The differences between the tensile strengths of the wounds among the various diet groups were not significant ($F < 1$). Considering only the groups sacrificed on the fourth day postinjury, comparison of the two groups standardized at minimal protein requirement (mean tensile strength 56 Gm.) with the other 3 groups (mean tensile strength 84 Gm.) does not reveal a significant difference either when this comparison is made from the over-all analysis of variance ($F < 1$) or when a separate analysis of variance is made for the fourth postinjury day alone ($F = 3.91$, $P > 0.05$). Similarly in separate analyses of variance for the seventh postinjury day ($F < 1$) and the tenth postinjury day ($F = 1.79$, $P > 0.05$) the differences between diets are not significant. The mean tensile strength of all diet groups rose from 73 Gm. on PI 4 to 322 Gm. on PI 7 and 445 Gm. on PI 10. In the over-all analysis of variance the differences between postinjury days are highly significant ($F = 157.55$, $P < 0.01$) and the individual differences between PI 4 and PI 7 and between PI 7 and PI 10 are also highly significant.

TABLE VI. WOUND TENSILE STRENGTH (MEANS AND STANDARD DEVIATIONS)

GROUP	PI 4 (GM.)	PI 7 (GM.)	PI 10 (GM.)
1-1	59 ± 34	372 ± 42	491 ± 124
1-2	53 ± 37	327 ± 97	520 ± 160
2-2	90 ± 39	323 ± 107	365 ± 94
3-3	86 ± 35	301 ± 61	417 ± 38
3-2	78 ± 31	296 ± 45	429 ± 56

In summary, no effect of protein level in the diet before injury or after injury on the tensile strength of the wound was observed.

Wound Histology.—The mean values of the scores obtained by histologic grading of the wounds are given in Table VII. In no instance are the differences between dietary groups significant. The hematoxylin and eosin and van Gieson stains gave steadily rising scores in all groups as the number of days postinjury increased, thus showing good correlation with the tensile strength measurements. The toluidine blue and leukofuchsin methods for mucopolysaccharides and glycoproteins showed decreasing intensity of staining with maturation, as has previously been reported by Pirani and associates.[10]

TABLE VII. MEAN SCORES OF WOUNDS GRADED HISTOLOGICALLY

STAIN	VAN GIESON			TOLUIDINE BLUE			LEUKOFUCHSIN		
POSTINJURY DAY	PI 4	PI 7	PI 10	PI 4	PI 7	PI 10	PI 4	PI 7	PI 10
Diet group									
1-1	1.6	2.6	4.0	3.2	2.2	1.0	2.2	2.0	1.0
1-2	1.2	2.5	4.0	3.4	2.8	1.2	2.0	2.3	1.0
2-2	1.8	2.8	3.5	2.2	1.6	1.8	1.2	1.7	1.2
3-3	1.4	3.0	4.0	2.7	1.7	1.0	1.8	1.7	1.2
3-2	1.4	2.5	3.8	3.2	2.0	2.0	1.3	1.7	1.2

The burns were examined histologically after staining with hematoxylin and eosin. No important differences between dietary groups were noted.

SUMMARY

Protein was fed to male rats at levels corresponding to one, two, or three times the minimum requirement (160, 320, or 480 mg. N per day) with calories sufficient to maintain body weight at 300 grams. These intakes in fourteen days produced uniformly positive nitrogen balance. At the end of this standardization period controls were sacrificed and the remaining animals subjected to a dorsal burn and a 4 cm. laparotomy. Recovery was followed for ten days, during which the groups were subdivided so that in each series one group was fed the same diet as during standardization and a second group the intermediate nitrogen level. Animals were killed on the fourth, seventh, and tenth postinjury days. Metabolic response was evaluated by urinary nitrogen excretion, liver composition, and adrenal weight and wound healing by tensile strength measurement and histologically.

None of the criteria employed detected significant alterations due to the level of protein fed prior to injury, except urinary nitrogen excretions and liver nitrogen content. In the groups prefed the minimal protein level, urinary excretion and hence negativity of nitrogen balance were less on the day of injury and first recovery day than in groups prefed the higher levels. The liver nitrogen content was higher at the end of the standardization period in the groups receiving the higher protein intakes than in the group receiving the minimal level.

During the recovery period, feeding higher levels of protein resulted in more positive nitrogen balance and greater total liver nitrogen content than did feeding the minimum requirement, but increasing the level from two to three times requirement afforded no further advantage. There were no other differences attributable to variation in protein intake after injury.

There were no significant differences attributable to level of protein feeding before or after injury in the tensile strength or histologic grading of the wounds.

CONCLUSIONS

It is concluded that maintenance of a high plane of protein nutrition before injury is without obvious benefit to the animal in terms of wound healing as compared with minimal, adequate nutrition.

Acknowledgment.—The chemical analyses reported here were performed by the Stability Division of the Food Laboratories, QMFCI. We are indebted to Mr. J. J. McMullen and the personnel of his testing group for these services. The assistance of Miss Mary Fox, Cpl. Robert Slechta, Mr. K. Sutherland, and Mr. F. Chereso of the Pathology Section, Medical Nutrition Laboratory, and of Mr. Lucius Thomas and Miss Gladys Eckfeldt of the Nutrition Division, QMFCI, in the performance of the experiment is gratefully acknowledged.

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